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We hypothesize that one can use specific protein or peptide sequences to direct bacteriophage vectors to dendritic cells. We further propose that one can then use such retargeted phage vectors to deliver potentially important antigens to dendritic cells, and that this may allow one to derive vectors capable of eliciting potent immune responses to breast cancer antigens such as her2. These hypotheses are being experimentally tested. During the period covered by this progress report, we have used phage display technology to identify peptide sequences which bind to cellular receptors expressed on dendritic cells, and we have conducted proof-of-concept studies to show that we can selectively target adenovirus vectors to dendritic cells using these peptide sequences. We have also shown that one can enhance intracellular internalization of T7 bacteriophage vectors using cell-binding peptides. We are now examining whether can use these same peptide sequences to deliver T7 and lambda phage vectors encoding a mammalian expression cassette, to primary dendritic cells. These experiments are expected to provide direct support for our hypothesis, that one can use phage vectors to express foreign genes in dendritic cells (including antigenic molecules, such as her2).

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Stefanie Jent 5/7/02

PI - Signature Date

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INTRODUCTION

The overall purpose of this application is develop a novel method for breast cancer vaccine development. The hypothesis which we propose is as follows: that dendritic cell (DC)-targeted bacteriophage vectors expressing a tumor antigen can be used to elicit specific and potent anti-tumor CD8+ T lymphocyte responses. The experiments being performed under the auspices of this grant award are intended to establish proof-of-concept for the approaches set forth in this application, which are expected to have strong potential for clinical translation and for application to other systems (e.g., retargeting of other virus vectors to DC). If successful, these studies could have important implications since phage vectors are simple and inexpensive to produce, highly stable and not hampered by problems of pre-existing immunity (unlike many mammalian viral vector systems).

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Development of phage vectors that target dendritic cells (months 1 - 15)
- *Task 2.* Generation of DC-targeted phage vectors that express an epitope from human HER-2/neu (DC-HER2 phage) (months 13-29)
- *Task 3.* Analysis of the immunogenicity of DC-HER2 phage (months 30-36)

Research Accomplishments associated with the above tasks

Task 1: Development of phage vectors that target dendritic cells

New findings are summarized on the following pages.

Development of phage vectors that target dendritic cells.

Generation of a T7 bacteriophage library displaying random peptides. We have constructed a random peptide display library in bacteriophage T7. This vector system is advantageous because (1) it grows faster than M13, (2) it expresses a high peptide copy number (415 copies per particle) and (3) because T7 phage has an isocahedral structure of a size that is very similar to that of many mammalian viruses. Thus, T7 phage is likely to be able to subvert pathways which mammalian viruses use to gain entry into mammalian cells.

A Cys-constrained heptapeptide T7 library comprising 2 x 10^8 independent clones was derived using commercially available reagents (Novagen). Twelve clones were then chosen at random for sequence analysis, which revealed that (as expected), each of the clones was different (i.e., independent).

Screening of T7 peptide display phage for the ability to enter human cells. We have developed methods for analysis and recovery of T7 phages which become internalized into mammalian cells. To further explore the utility of this technology, we compared the ability of three different phage populations to undergo internalization into immortalized human endothelial cells (HCEC cell line). For this experiment, we examined internalization by (1) an unselected peptide-display T7 library (i.e., a diverse phage population, comprising 2 x 10^8 independent clones), (2) a randomly selected clonal peptide-display T7 phage population, and (3) a peptide-display T7 phage population selected for the ability to bind to purified recombinant $\alpha_v\beta_3$ integrin (selection was for one round only, so this population is expected to be quite diverse and by no means optimized for integrin-binding). The results, which are shown in Fig. 1, reveal that the integrin-selected phage population has a 3-fold enhanced ability to enter HCEC cells, as compared to the starting phage population (the library), and a 10-fold enhanced ability to enter HCEC cells, relative to a negative control phage clone. Thus, even a partial selection for the ability to bind an endocytosing cell surface receptor (such as an integrin) resulted in a significant increase in the ability of T7 phage to enter human cells. ***These results (Fig. 1) were previously reported as Preliminary Data in our initial grant application.***

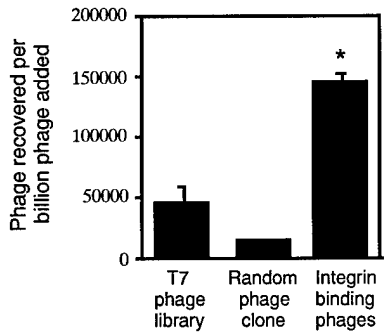


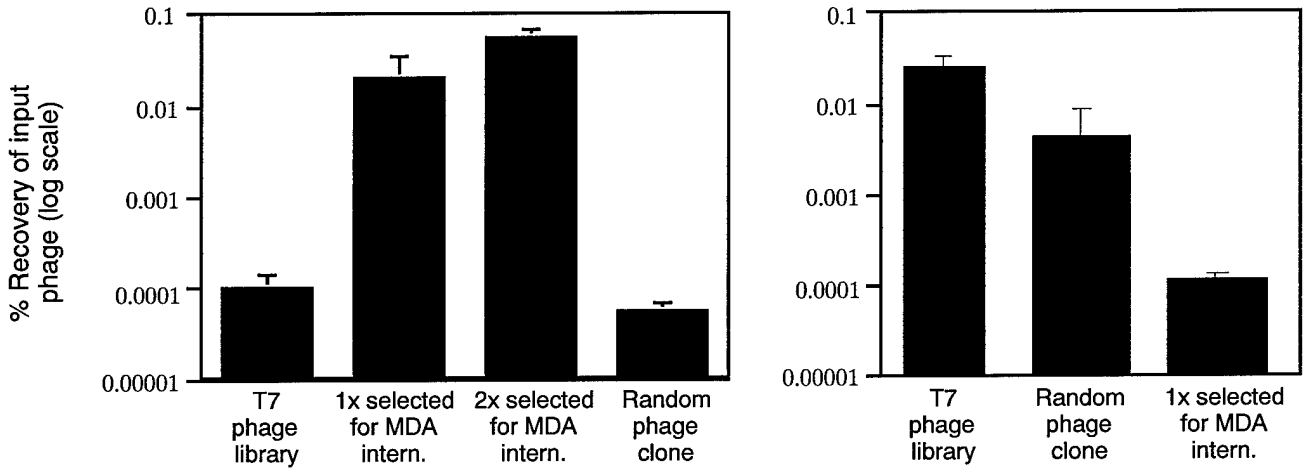
Figure 1: Phage Internalization in human endothelial cells. Equal amounts (10^9 pfu) of each phage population were added to human endothelial cells (HCEC), and phage was allowed to internalize at 37°C. Cell-bound phage was then removed by extensive washing and proteolytic digestion, and internalized phage was rescued by lysis of the cells, and infection of *E. coli*. Phage titers were then calculated. See text for description of the phage populations that were screened. *: Statistically significant difference from other groups ($p < 0.005$).

As shown above, the integrin-binding phage population exhibited roughly a 3-fold enhancement in the ability to enter mammalian cells, relative to an unselected phage population. This suggested that further enrichment for integrin-binding phage might result in an even better level of cell entry. Unfortunately this was not found to be the case.

Briefly, we were able to successfully select phage clones from our random phage display libraries, which possessed the ability to bind to purified cellular integrins, including integrins that are highly expressed on dendritic cells, such as the alpha$_v$beta$_3$ ($\alpha_v\beta_3$) integrin (data not

shown). These integrin-binding phage clones did not, however, undergo efficient internalization into mammalian cells, under any experimental conditions tested (data not shown).

We therefore pursued an alternative strategy, in which we directly screened random phage display libraries for the ability to undergo internalization into mammalian cells. This was made possible by the development of efficient washing and protease-digestion methods, which were able to eliminate non-specifically bound or extracellular phage —thereby allowing us to enrich for internalized (protease resistant) phage. Using this approach, we were able to select for phage populations which were enhanced in their ability to enter a particular mammalian cell line (MDA-MB-231 breast cancer cells; Figure 2, left hand panel). Strikingly, however, these selected phage populations were <u>not</u> enhanced in their ability to enter a second breast cancer cell line (SK-BR-3; Figure 2, right hand panel).



<u>Figure 2: Phage Internalization in human breast cancer cells</u>.
<u>Left Panel: Internalization into MDA-MB-231 breast cancer cells</u>: Phage populations were as follows: Phage selected for their ability to enter human breast cancer cells (MDA-MB-231; abbreviated MDA), either for one round (1x) or two rounds (2x), as well as the original unselected library (T7 phage library) or a randomly chosen phage clone. Phages were added to MDA-MB-231 cells, and 4 hours later, internalized phage was quantitated by performing phage plaque assays using lysates that were prepared from extensively washed cell monolayers that were also subjected to protease digestion to remove non-specifically bound phage. It can be observed that we were able to successfully select for phage clones which had an enhanced ability to enter MDA-MB-231 cells.
<u>Right Panel: Internalization into SK-BR-3 breast cancer cells</u>: Phage populations were as follows: Phage subjected to one round of selection for their ability to enter MDA-MB-231 human breast cancer cells (abbreviated MDA), as well as the original unselected library (T7 phage library) or a randomly chosen phage clone. Phages were added to SK-BR-3 human breast cancer cells, and 4 hours later, internalized phage was quantitated as described above. It can be observed that phage clones which had an enhanced ability to enter MDA-MB-231 cells did NOT have an enhanced ability to enter the closely related SK-BR-3 cell line.

The data shown in Figure 2 suggest that direct selection for phage entry into a particular cell line (MDA-MB-231 cells in this case) does not necessarily result in the derivation of a phage population that can successfully enter another cell line, even if that cell line is species-matched and of the same origin (SK-BR-3 cells and MDA-MB-231 cells are both human breast cancer cell lines). This result suggests that it may be somewhat naive to expect that

selection for phage clones which enter a murine dendritic cell line (XS52 cells) will result in the identification of phages capable to entering primary human dendritic cells. With this in mind, we decided to concentrate our efforts on the biopanning of random phage display libraries against well-defined protein antigens that are known to be expressed on the surface of dendritic cells.

Ligand-based selection of phage display libraries. As noted in the preliminary data that were included in our original grant application, we have been able to successfully screen both M13 and T7 random phage display libraries for the ability to bind to immobilized, purified murine CD40 (as shown in Figure 3).

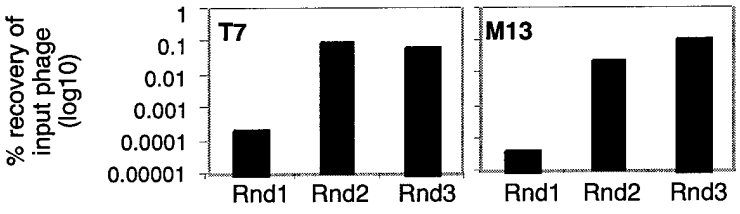


Figure 3: Biopanning of peptide phage display libraries using recombinant murine CD40 (CD40:Ig). Two phage display peptide libraries (constructed in phage T7 or M13) were subjected to 3 rounds of biopanning against muCD40:Ig. The recovery of bound phage is shown for each round of panning (expressed as a percentage of total input phage)

Subsequent sequence analysis of randomly selected phage clones amplified after the third round of biopanning revealed that a consensus peptide sequence had been selected (sequence = FN[G/P][N/S], which was present in 4/5 clones that were sequenced). This consensus motif (FNGP) was also present in the immunoglobulin heavy chain variable region from a CD40-specific mouse monoclonal antibody (GenBank accession AAB81501), suggesting that it may be biologically significant. Since this monoclonal antibody is capable of delivering a covalently linked molecule into the cytosol of CD40-positive target cells (4), this observation also suggests that one or more of the phage-selected peptides may be capable of mediating phage or virus internalization into target cells. In light of this possibility, we therefore examined whether the phage clones selected for the ability to bind to CD40 might be capable of entering primary dendritic cells, which are known to express CD40. Results from these experiments are shown below (Figure 4). The data show that phage populations which are enriched in clones with the ability to bind to CD40 can indeed enter primary dendritic cells with as much as 100-fold greater efficiency than the initial, non-selected phage population (compare the 2x CD40-selected population in the right hand panel of Figure 4 to the unselected starting phage population in the left hand panel of Figure 4).

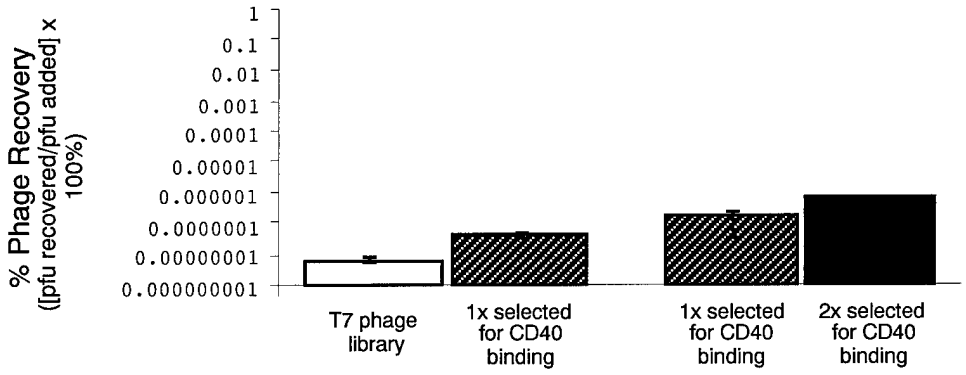


Figure 4: Internalization of CD40-binding phage populations in primary murine dendritic cells. Phage populations were as follows: Phage selected for their ability to bind to purified recombinant murine CD40 (mCD40), either for one round (1x) or two rounds (2x) of biopanning, as well as the original unselected library (T7 phage library). Results from two separate experiments are shown; the leftmost two bars represent a comparison of the 1x selected phage population to control phage, while the rightmost two bars represent a comparative analysis of internalization by the 1x versus the 2x selected phage population. Phage populations enriched in CD40-binding clones were also enriched with respect to the ability to enter primary dendritic cells, and 2x selected population was roughly 100-fold better at entering primary dendritic cells than the starting phage population. *Method: Phages were added to primary murine dendritic cells (derived from murine bone marrow, by cultivation in the presence of interleukin-4 and GM-CSF), and 3 hours later, internalized phage was quantitated as described in the legend to Fig. 2.*

Use of the Tat protein transduction domain for phage targeting to dendritic cells. A peptide derived from HIV-1 Tat, known as the Tat protein transduction domain (PTD) peptide, has previously been shown to be capable of enhancing macromolecular delivery into mammalian cells via an endosome- independent pathway that appears to depend on engagement of cell surface heparan sulfate proteoglycans (HSPGs) (3, 5, 15). We have therefore constructed T7 phage clones which express this Tat PTD peptide on their surface, and we have examined their ability to undergo internalization in mammalian cells. The results of these experiments have revealed that the Tat PTD peptide strongly enhances the internalization of T7 phage in mammalian cells (Figure 5).

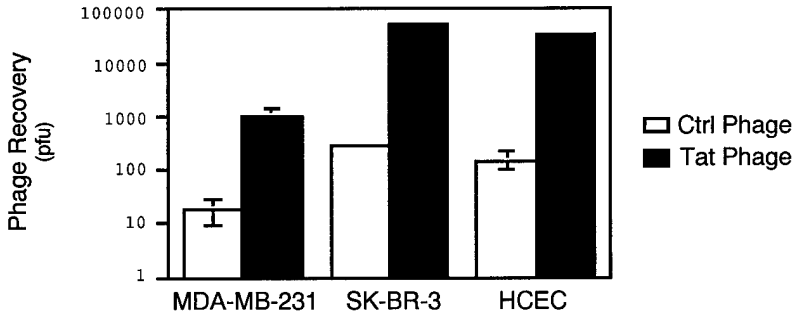


Figure 5: Internalization of T7 phage clones that display the Tat PTD peptide, in mammalian cells. Phage populations were as follows: Phage which display the Tat PTD on their surface (Tat), as well as a randomly selected phage display clone (Ctrl). 10^{11} phages were added to the indicated cell lines (MDA-MB-231, SK-BR-3: human breast cancer cell lines; HCEC: human brain endothelial cell line) and 3 hours later, internalized phage was quantitated as described in the legend to Fig. 2. Tat PTD-displaying phage clones were greatly enhanced in their ability to enter mammalian cells, as compared to unmodified phage clones.

Retargeting of adenovirus to dendritic cells. Our primary aim in the present set of studies is to derive novel methods for the targeting of phage vectors to mammalian cells. However, it may also be possible to use phage-selected peptides to target mammalian virus vectors to dendritic cells. This could enhance the utility of the approaches we are taking.

With this in mind, we have performed preliminary experiments to examine the feasibility of retargeting adenovirus vectors to murine dendritic cells, using peptide sequences that were derived from CD40-binding phage clones. The rationale for these studies was that adenovirus vectors are normally very inefficient in terms of their ability to transduce primary dendritic cells (1, 13); thus, virus retargeting could substantially enhance the ability of these vectors to infect dendritic cells (18).

For these experiments, bifunctional oligopeptides were synthesized, containing two distinct domains, separated by a short spacer (GGGS). Functional peptide domains were as follows: (1) a motif that binds to the adenovirus fiber protein (MH20; RAIVGFRVQWLRRYFVNGSR (6)), and (2) a phage-selected peptide derived from the CD40 biopanning experiment (ATYSEFPGNLKP). The peptides were then added to target cells (bone marrow-derived murine dendritic cells, matured in the presence of IL-4 and GM-CSF), and the cells were then exposed to a fixed amount of an adenovirus vector expressing the jellyfish green fluorescent protein (Ad:GFP). GFP expression in the cell population was then quantitated 48 hours later, using flow cytometry. The results of this experiment are shown in Figure 6. The data reveal that CD40-selected peptides can indeed enhance adenovirus infection of primary murine dendritic cells.

Very similar results were also obtained when these same peptides were used to retarget the Ad:GFP vector to primary human dendritic cells (Figure 7).

Taken together, these results suggest that peptides identified through biopanning of phage display libraries may not only have utility for directing cell entry by bacteriophage vectors, but they may also allow one to redirect mammalian virus vectors to primary dendritic cells.

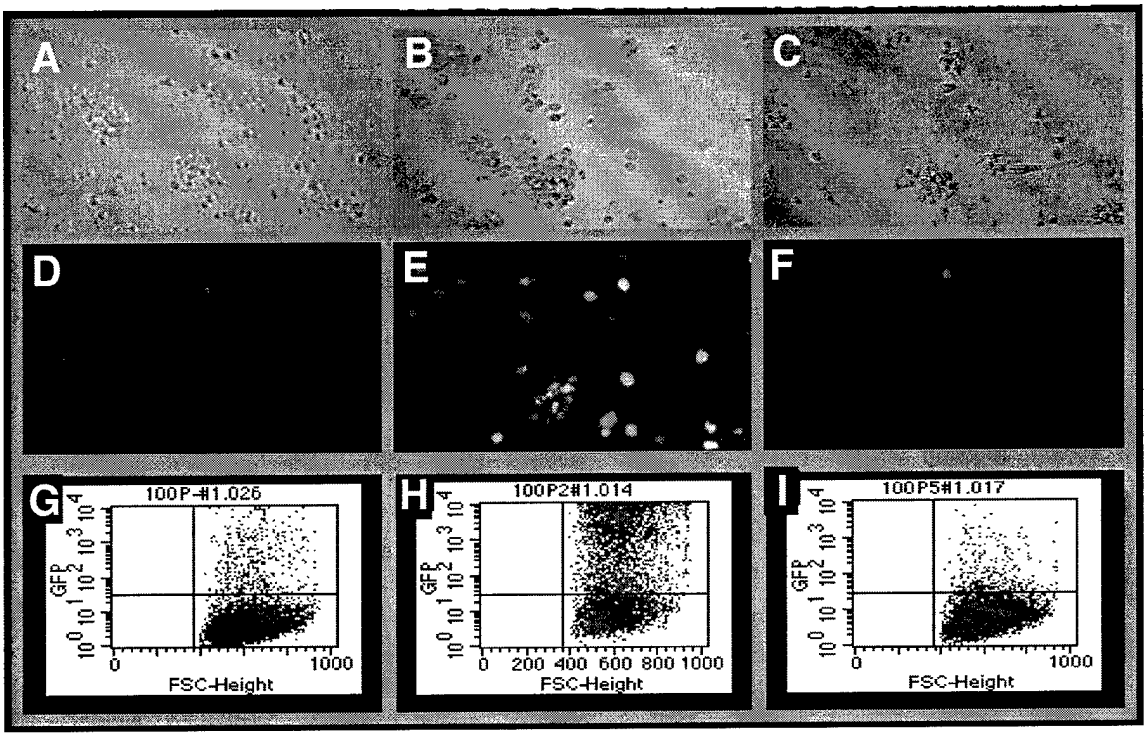


Figure 6: Infection of primary murine dendritic cells by a GFP-expressing adenovirus vector, following conjugation to a putative CD40-binding peptide. **(A, B, C):** Bright field images of primary murine dendritic cells; **(D, E, F):** Fluorescence images of the same cells, at 48 hours following infection with a fixed inoculum of a GFP-expressing adenovirus (Ad:GFP), in the absence of any exogenous peptide (D), in the presence of a CD40-binding bifunctional peptide (E), or in the presence of a mutated derivative of the CD40-binding peptide that lacks the FPGN consensus motif (F). **(G, H, I):** Flow histograms for GFP fluorescence (Y axis), plotted against cell number (X axis) for the same cell populations shown in panels D through F, respectively. It is readily apparent that there is little GFP expression in cells that were exposed to the Ad:GFP reagent in the absence of any exogenous peptide (G) or in the presence of the mutated peptide (I); in contrast, there is strong GFP expression in cells which received Ad:GFP in the presence of the bifunctional peptide that contains the CD40-binding motif. *Note: Bifunctional peptides were as follows: ATYSEFPGNLKPSGGGRAIVGFRVQWLRRYFVNGSR (CD40-binding) and FKEAGSPYTLPNGGGRAIVGFRVQWLRRYFVNGSR (mutated control peptide). Adenovirus was preincubuated with peptide in a volume of 20 l in culture medium (RPMI-1640 medium with 10% fetal bovine serum plus 1% donor serum) for 30 min., prior to addition to dendritic cells (100,000 cells) at a multiplicity of infection of 200.*

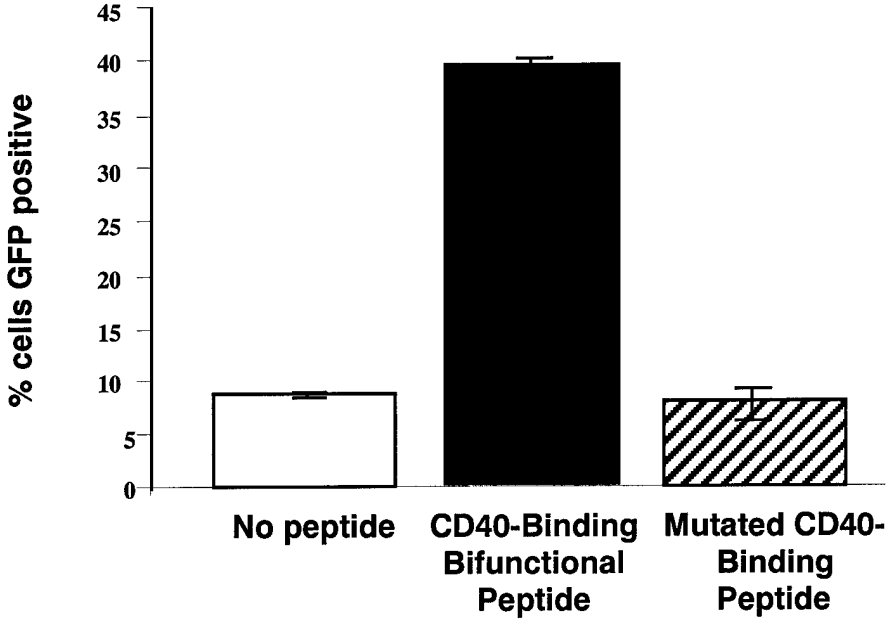


Figure 7: Infection of primary human dendritic cells by a GFP-expressing adenovirus vector, following conjugation to a putative CD40-binding peptide. Primary human dendritic cells (differentiated from CD14+ blood monocytes in the presence of in IL-4 and GM-CSF) were exposed to a fixed inoculum of a GFP-expressing adenovirus (Ad:GFP), in the absence of any exogenous peptide (no peptide), in the presence of a CD40-binding bifunctional peptide, or in the presence of a mutated derivative of the CD40-binding peptide that lacks the FPGN consensus motif. The percentage of GFP positive cells within the cultures was then determined by flow cytometric analysis 48 hours later. It is readily apparent that there is little GFP expression in cells that were exposed to the Ad:GFP reagent in the absence of any exogenous peptide or in the presence of the scrambled (mutated) peptide; in contrast, there is strong GFP expression in cells which received Ad:GFP in the presence of the bifunctional peptide that contains the CD40-binding motif. *Note: Bifunctional peptides and experimental conditions were exactly as described in the legend to Figure 6.*

Current efforts to generate phage vectors that target dendritic cells. As noted in our original grant application, the filamentous bacteriophage M13 has been used to deliver exogenous DNA to mammalian cells (8, 11). Thus, it appears feasible to use phage vectors to deliver genes into mammalian cells (including dendritic cells). However, phage M13, the most widely used phage vector in mammalian gene delivery experiments, is a rigid rod that is roughly 10-fold longer than most mammalian viruses. Thus, M13 may be inherently ill-suited for the purpose of gene delivery to mammalian cells. In contrast, bacteriophage T7 or phage lambda may be much better suited for this purpose, since they are similar in size and shape to common mammalian viruses.

T7 bacteriophage is particularly appealing because of its widespread usage (7, 16), rapid growth in culture, convenience and ability to allow high copy number peptide expression (415 copies per particle). We have therefore devoted considerable time and enery during the present project year to the derivation of a T7 clone which contains a readily detectable mammalian expression cassette (a promoter and poly A sequence, flanking the GFP indicator gene). The utility of such a GFP-expressing phage is that it would provide us with a direct way to examine not only phage entry into mammalian cells, but also phage uncoating —since both

entry and uncoating would be necessary for GFP expression. This important advantages in terms of testing some of the hypothesis set forth in this grant.

Unfortunately, the construction of a T7 phage clone which incorporates a mammalian GFP expression cassette has proven more difficult that we had anticipated. This is in part because T7 is a highly recombinogenic phage. Thus, we have found that the phage readily deletes or rearranges inserted foreign DNA, if that DNA has even very short stretches of repetitive sequence (data not shown). While somewhat unexpected in light of the common use of T7 phage as a scaffold for protein or peptide display, this finding is in fact consistent with previous reports concerning the recombinogenic nature of T7 phage genomes and their propensity to undergo deletional mutagenic events (9, 10, 14, 20, 21). This may be a consequence of the recombination-based replicational mechanism employed by T7 phage.

Whatever the reason for these unexpected difficulties, we are nonetheless continuing to try to insert a mammalian expression cassette into the T7 genome, using different promoter and polyA elements and also different indicator genes (luciferase, GFP). At the same time, we have also initiated collaborative efforts with Dr. Ron Hoess (DuPont), Dr. Andreas Plueckthun and others, with the intention of deriving retargeted lambda phage vectors. We believe that this approach has considerable merit for several reasons: (1) lambda is very similar in size and shape to T7 phage (i.e., it is similar to mammalian viruses); (2) lambda is widely used for cloning and expression of all manner of genes, and lambda phage vectors containing mammalian transcriptional regulatory elements (promoter, poly A) are commercially available (Stratagene); (3) lambda vectors have previously been adapted so as to enter mammalian cells (2, 3); and (4) lambda vectors can be conveniently employed as a scaffold for moderate to high copy (up to approximately 400 copies per capsid) display of short peptides or even large proteins, when such molecules are fused in frame to the lambda capsid protein, gpD (12, 17, 19). In fact, gpD is highly unusual in its ability to readily tolerate very large additions at either the N- or C- terminus, with no apparent effects on protein structure, function or protein copy number on the phage capsid (19); these properties are significantly different from those of the T7 major coat protein which can tolerate large inserts but only if the modified protein is present at very low copy number in the assembled phage capsid.

Fusion of candidate targeting peptides to the terminus of lambda phage gpD also provides one additional advantage in terms of our proposed experiments. Specifically, by amplifying a wild-type lambda phage genome, or a lambda phage genome bearing a reporter gene cassette, in bacterial cells which constitutively or inducibly express gpD, one can conveniently generate phage progeny which are chimeric in terms of the composition of gpD molecules in their capsids. Such phage will contain a mixture of wild-type gpD molecules (derived from the phage genome) and recombinant gpD molecules (derived from the E. coli host strain). Since the addition of large inserts to the terminus of gpD has little effect on protein function or capsid incorporation, it is therefore possible to readily obtain chimeric phage capsids in which 20-50% of the gpD molecules are of recombinant origin; this corresponds to approximately 40 to 200 recombinant gpD molecules per phage capsid (19). We are presently cloning our CD40-targeting peptides, as well as additional peptides, onto the terminus of gpD so as to allow us to produce gpD-chimeric lambda phage particles, targeted to desired cellular receptors. These lambda particles will be generated using recombinant phage genomes which contain mammalian expression cassettes (luciferase, β-galactosidase) so as to allow us to evaluate directly whether we can successfully retarget these vectors into dendritic cells, using recombinant, modified gpD proteins.

Tasks 2, 3: These tasks remain to be initiated (as per the original statement of work)

KEY RESEARCH ACCOMPLISHMENTS

- Development of methods which allow us to (A) quantitatively assess bacteriophage internalization into mammalian cells, and (B) select for bacteriophage clones which are enhanced in this property.

- Selection of peptides capable of binding to the CD40 receptor, using phage display technology. In proof-of-principle experiments, these peptides have been shown to enhance the infection of primary murine and human dendritic cells by a mammalian virus vector (adenovirus) when non-covalently conjugated to the surface of that vector. We have also shown that phage populations enriched in CD40-binding clones undergo internalization into primary dendritic cells with approximately 100-fold greater efficiency than control phage.

- Generation of T7 vectors which express the HIV-1 Tat protein transduction domain (PTD) peptide on their surface, and demonstration that such vectors undergo internalization into mammalian cells at an enhanced rate, as compared to control phage.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: None, although we are presently in the process of writing up some of the results from our CD40-targeting project, for submission to a suitable journal later this year.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Ms. Rebecca Wiley was provided (Ms. Wiley is a 2001 college graduate, who has been working on this project as a laboratory technician). Ms. Wiley will attend graduate school next fall, to obtain her Ph.D. in Immunology, and the present research experiences will undoubtedly assist her in that goal. Research training for an undergraduate researcher, Mr. Aaron Merriam, has also been provided. Mr. Merriam s long-term goals remain uncertain, since he is starting freshman at the University of Rochester. However, his goals will likely include graduate (Ph.D. or M.D.) school — goals which will be enhanced by his training under this award.

CONCLUSIONS

The conclusions which can be drawn from the first year of our experiments are as follows:

1. It is possible to directly screen random peptide display libraries constructed in T7 bacteriophage for the abliity to undergo internalization into mammalian cell types of interest, by using a combination of extensive washing and proteolytic degradation to eliminate extracellular and surface-bound phage, respectively.

2. We have identified CD40-binding peptides by screening random peptide display libraries, and we have shown in proof-of-concept studies that these peptides can be used to enhance the infection of primary murine and human dendritic cells, when non-covalently coupled to the surface of a readily detectable mammalian virus vector (an adenovirus vector that expresses green fluorescent protein). We have also shown that phage populations which are enriched in CD40-binding clones undergo internalization into primary dendritic cells with roughly 100-fold greater efficiency than control phage.

3. We have generated recombinant T7 bacteriophage clones which express a short protein transduction domain (PTD) peptide from HIV-1 Tat on their surface, and we have shown that these PTD-bearing phage clones are capable of efficiently entering cultured mammalian cells.

<u>So What Section</u>

The knowledge gained from these experiments advances the basic goals of this grant application, and brings us closer to being able to test our underlying hypothesis, that genetically modified bacteriophage vectors may represent a useful system for permitting gene (antigen) transfer to dendritic cells, for purposes of breast cancer vaccine development.

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APPENDICES

None